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Serological studies of *Bacteroides fragilis*

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SUMMARY

Using direct agglutination methods, a simple serological scheme for the classification of *Bacteroides fragilis* is described. Twenty strains of *B. fragilis* were selected by a process of successive screening from 151 strains obtained from various sources. O-antigens were prepared from the 20 strains, and used to raise antisera in rabbits.

Each of the 20 antisera reacted with its homologous antigen and eight antisera cross-reacted with other subspecies. These cross-reactions were successfully removed after absorption of the antisera with the cross-reacting antigens, resulting in 19 type-specific antisera, titres ranging from 40 to 320, and 19 distinct serotypes of *B. fragilis*. There was no correlation between the antigenic and the biochemical characteristics of these strains and no cross-reactions occurred with other gram-negative anaerobes, *B. melaninogenicus*, *Sphaerophorus necrophorus* and *Fusobacterium necrogenes*.

INTRODUCTION

Bacteroides fragilis is the commonest anaerobic organism associated with clinical infections in man (Finegold, 1968, 1974; Gorbach & Bartlett, 1974; Mitchell, 1973). The identification and classification of *Bacteroides* spp. have been based on morphological and cultural characteristics and the products of glucose metabolism, as well as other biochemical reactions (Cato *et al.* 1970; Holdeman & Moore, 1972). On this basis Holdeman & Moore (1974) divided *B. fragilis* into five subspecies. *B. fragilis fragilis*, *B. fragilis thetaiotaomicron*, *B. fragilis vulgatus*, *B. fragilis distasonis* and *B. fragilis ovatus*. However, the frequent variations of the biochemical reactions make the interpretations difficult and alternative methods of classification have been used.

Since the antigenic structure of bacteria is stable, attempts at classification using serological techniques were investigated. Previous workers have demonstrated the existence of species-specific lipopolysaccharide antigens among different *Bacteroides* species tested (Sonnerwith, 1960; Werner, 1969; Sharpe, 1971). Beerens, Wattre, Shinjo & Romond (1971) on the other hand, using antisera against the five subspecies described by Holdeman & Moore (1974), classified 131 strains into six major and several minor serogroups. More recently Lambe & Moroz (1976)

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classified *B. fragilis* subsp. *fragilis* into 21 serogroups, seven of which were specific and the remainder consisted of variable numbers of components.

In this work we set out to produce antisera to a number of *Bacteroides fragilis* strains derived from multiple sources, and to use a simple serological scheme based on the somatic antigen, similar to those existing for *Escherichia coli* and other Enterobacteriaceae (Kauffmann, 1947). Such a scheme will help in determining the relation between the antigenic and the biochemical characteristics of the *B. fragilis* group, and will provide a basis for understanding the role of these bacteria in health and disease.

MATERIALS AND METHODS

Bacterial strains

For the purpose of this study, four strains of *B. fragilis* from the National Collection of Type Cultures (*B. fragilis* subsp. *fragilis* NCTC 9343, *B. fragilis fragilis* NCTC 10584, *B. fragilis thetaiotaomicron* NCTC 10582 and *B. fragilis vulgatus* NCTC 10583) were designated as O1–O4 and used to raise antisera. These antisera were tested against another five strains (*B. fragilis fragilis* NCTC 9344, *B. fragilis fragilis* NCTC 8560, *B. fragilis fragilis* NCTC 10581, *B. fragilis ovatus* ATCC 8483, *B. fragilis distasonis* ATCC 8503) and showed no agglutination reactions. These five strains were therefore designated O5–O9 and used to raise further antisera.

The nine antisera thus obtained were tested against 25 strains of *B. fragilis* isolated from clinical specimens. Fourteen strains gave negative reactions. Six of those and one strain (O11) which gave cross-reactions, were designated as O10–O15a and used to raise further antisera.

All the 16 antisera were then tested against 117 strains of *B. fragilis* obtained from clinical specimens. Thirty-three strains gave negative reactions. Three of them and one (O16) which gave cross-reactions were designated as O16–O19.

The source and the subspecies of the 20 strains (O1–O20), described above, are shown in Table 1.

B. melaninogenicus NCTC 9336, *Sphaerophorus necrophorus* NCTC 19575 and *Fusobacterium necrogenes* NCTC 19723, obtained from the National Collection of Type Cultures, were also included in the study.

Culture media

DST agar (Oxoid) 4 % (w/v), lysed horse blood 5 % (v/v), incorporating Gentamicin Sulphate 0·01 % (w/w) was used to grow all micro-organisms.

Peptone yeast broth prepared as described by Cato *et al.* (1970) was used to culture *Bacteroides* strains for the production of antigen.

Identification of B. fragilis

All isolates from clinical laboratory specimens were checked for purity, and aerobic and anaerobic growth, before being submitted for identification. They were identified to the genus level on the basis of their colonial appearance, cell morphology (Gram stain) as well as the detection of the end products of glucose

Table 1. *The strains of* Bacteroides fragilis, *their source and O-antigen*

O-antigen	Strains of *Bacteroides fragilis* (*B. f.*)	Source of isolates
O1	*B. f. fragilis* NCTC 9343	
O2	*B. f. fragilis* NCTC 10584	
O3	*B. f. thetaiotaomicron* NCTC 10582	
O4	*B. f. vulgatus* NCTC 10583	
O5	*B. f. fragilis* NCTC 9344	
O6	*B. f. fragilis* NCTC 8560	
O7	*B. f. fragilis* NCTC 10581	
O8	*B. f. ovatus* ATCC 8483	
O9	*B. f. distasonis* ATCC 8503	
O10	*B. f. fragilis*	Cholecystectomy wound
O11	*B. f. fragilis*	Appendicectomy wound
O12	*B. f. fragilis*	Peritonitis – exudate
O13	*B. f. distasonis*	Faeces
O14	*B. f. distasonis*	Faeces
O15	*B. f. distasonis*	Small intestinal tissue – Crohn's disease
O15a	*B. f. vulgatus*	Small intestinal tissue – Crohn's disease
O16	*B. f. fragilis*	Appendicectomy wound
O17	*B. f. fragilis*	Cholecystectomy wound
O18	*B. f. thetaiotaomicron*	Pilonidal sinus
O19	*B. f. distasonis*	Exudate – infected umbilicus

metabolism by gas liquid chromatography as described by Cato *et al.* (1970). The strains of *B. fragilis* were further identified and assigned to subspecies on the basis of their biochemical reactions tested by the AP1 20A anaerobe system (Starr, Thompson, Dowell & Balows, 1973; Holdeman & Moore, 1972).

Preparation of O-antigens

Pure cultures of *B. fragilis* (Table 1) were grown for 48 h in 30 ml of peptone yeast broth (Cato *et al.* 1970). This provided a density of growth of approximately 4×10^8 bacteria per ml. The cultures were steamed for 30 min at 100 °C and centrifuged. The bacterial deposit was washed three times and resuspended in buffered physiological saline (Werner, 1972) and adjusted to give a reading similar to No. 5 Wellcome Opacity tube (Wellcome Reagents Limited).

Preparation of antisera

New Zealand white rabbits, weighing approximately 2 kg, were used for the preparation of the antisera. Before inoculation with the antigen suspension, 10 ml of blood were drawn from each rabbit in order to obtain control serum.

The immunization consisted of six intravenous injections of antigen suspension over a period of 6 weeks in increasing doses as follows: 1st week 0·25 ml; 2nd week 0·5 ml; 3rd week 1·0 ml; 4th week 2·0 ml; 5th week 2·0 ml; 6th week 2·0 ml.

Three days after the final injection, 40 ml of blood were withdrawn from the marginal vein of the ear of the rabbit. Subsequently 60 ml were withdrawn twice at 3–4 days interval. Four days after the last bleed the rabbit was exsanguinated

by cardiac puncture. The serum removed from the four samples was pooled and stored at −20 °C.

Testing of O-antisera

Tube agglutination methods were used. Each antiserum was tested against suspensions of its homologous antigen, and all the other antigens, as well as antigens prepared from *B. melaninogenicus* NCTC 9336, *Sph. necrophorus* NCTC 19575 and *Fus. necrogenes* NCTC 10723. The control sera were also tested against all antigens in order to exclude any pre-existing antibodies.

Each serum was diluted at 1/10 (v/v) in physiological saline. Merthiolate was added as a preservative giving a final concentration of 1/10000 (w/v). To a series of 0·2 ml volumes of each diluted serum were added equal amounts of each O-antigen suspension, giving a final dilution of 1/20 (v/v). These were incubated at 50 °C for 18 h and kept for a further 24 h at 4 °C.

Agglutination, read visually, was considered to have occurred when there was definite clumping at the bottom of the tube and the supernatant solution was completely clear and on tapping the agglutinated clumps were visible. Control tubes of saline mixed with O suspensions appeared uniformly turbid after similar treatment (Bettelheim, 1969). The O suspensions giving agglutination at such dilution were then tested against doubling dilutions of the antiserum and those showing no reaction at final dilution of 1/20 (v/v) were considered as negative.

Preparation of pure O-antiserum

Pure antisera were obtained by absorption with any cross-reacting antigens at 50 °C for 2 h, at a serum dilution of 1/10 (v/v). The sera were then centrifuged at 3000 rev./min for 30 min (Kauffmann, 1944; Bettelheim, 1969).

The absorbed sera were tested against all antigens with which the original sera had reacted. If they reacted only with homologous antigen, then a pure specific antiserum was considered to have been produced. Final titres were obtained by testing each antigen against doubling dilutions of its homologous absorbed serum.

RESULTS

The control sera showed no agglutination when tested against all the antigen suspensions. The agglutination results of the unabsorbed antisera tested against all antigen suspensions are shown in Table 2. All the O-antisera reacted with their homologous antigens and some of them also agglutinated heterologous antigens. The titres ranged from 20 to 320, with homologous reactions being generally higher than the heterologous reactions. No agglutinations were obtained against the antigen suspensions of *B. melaninogenicus*, *Sph. necrophorus* and *Fus. necrogenes*.

Table 3 illustrates in detail the agglutination reactions of O4, O15 and O15a antisera against absorbed and unabsorbed O4, O15 and O15a antisera. After absorption with O4 antigen, O15a antiserum reacted with both its homologous and O15 antigens, but when absorbed with O15 antigen, it failed to agglutinate O4, O15 as well as its homologous antigen.

Table 2. *Summary of homologous and heterologous agglutination reactions of 20 strains of* Bacteroides fragilis *and tests of their O-antisera against* B. melaninogenicus, Sphaerophorus necrophorus *and* Fusobacterium necrogenes

		Antisera against																				
	O-antigen	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14	O15	O15a	O16	O17	O18	O19	Sal. con.*
B. f. fragilis NCTC 9343	O1	40																		40		
B. f. fragilis NCTC 10584	O2		40									80						40				
B. f. thetaiotaomicron NCTC 10582	O3			40																		
B. f. vulgatus NCTC 10583	O4				40											80	40					
B. f. fragilis NCTC 9344	O5					40															80	
B. f. fragilis NCTC 8560	O6						320															
B. f. fragilis NCTC 10581	O7							320														
B. f. ovatus ATCC 8483	O8								40													
B. f. distasonis ATCC 8503	O9									160												
B. f. fragilis	O10										160											
B. f. fragilis	O11		20									320						40				
B. f. fragilis	O12							160					160									
B. f. distasonis	O13													40								
B. f. distasonis	O14														80							
B. f. distasonis	O15															160	80					
B. f. vulgatus	O15a															40	40					
B. f. fragilis	O16		20															80				
B. f. fragilis	O17																		160			
B. f. thetaiotaomicron	O18	20										20								320		
B. f. distasonis	O19																				160	
B. melaninogenicus NCTC 9336																						
Sph. necrophorus NCTC 10575																						
Fus. necrogenes NCTC 10723																						

* Saline control.

Table 3. *Agglutination reactions of O14, O15 and O15a antigens against unabsorbed and absorbed O4, O15 and O15a antisera*

		Antisera against							
		O4	O15				O15a		
				Absorbed with antigen				Absorbed with antigen	
Antigens		Unabsorbed	Unabsorbed	O4	O15a	O4+O15a	Unabsorbed	O4	O15
B. f. vulgatus NCTC 10583	O4	40	80	—	40	—	40	—	—
B. f. distasonis	O15	—	160	80	160	80	80	40	—
B. f. vulgatus	O15a	—	80	—	—	—	40	40	—

The agglutination results of the absorbed antisera tested against all the antigen suspensions are shown in Table 4. All the non-specific agglutinins were successfully removed after absorption of the antisera, and the final titres of the pure antisera ranged from 40 to 320.

DISCUSSION

This work demonstrates that *B. fragilis* forms a serologically heterogeneous group. It was possible to raise specific antisera against 20 strains derived from various sources. The nine strains (O1–O9) obtained from the National Collection of Type Cultures gave distinct and specific antisera. The remainder of the antisera were prepared from antigens (O10–O19) which were obtained by screening 142 various strains isolated from clinical specimens (Table 1). This suggests that screening more strains of *B. fragilis* isolated from clinical specimens and normal human faeces will provide new antigens, which can be used to extend this serological scheme.

The *B. fragilis* O-antigens used in this work consisted of the thermostable lipopolysaccharide (LPS) moiety of the cell wall. These allow the detection of strain and of species-specific antigen, whereas the common group or genus antigens are not demonstrable. Thus there were no cross-reactions between the *B. fragilis* group and the other non-sporing anaerobes tested, *B. melaninogenicus*, *Sph. necrophorus*, and *Fus. necrogenes* (Table 2).

Bacteroides O-antigens, however, give poor antibody response and the titres, demonstrated by direct tube agglutination tend to be low (Werner, 1969; Sharpe, 1971; Lambe & Moroz, 1976). This poor antigenicity of *Bacteroides* may be due to the different chemical composition of their LPS, as demonstrated by Kasper (1976).

High titres were reported when the antigens mixed with adjuvants were used to immunize the animals, but this ultimately resulted in numerous cross-reactions (Sonnerwith & Rosebury, 1960). In our work the titres obtained ranged from 20 to 320 with only minor cross-reactions occurring between eight of the 20 strains used (Table 2). This may be due to the presence of an antigen common to the species. Cross-reactions occurred between *B. fragilis* subsp. *fragilis* (O1), and *B. fragilis* subsp. *thetaiotaomicron* (O18) (Table 2) and also between *B. fragilis* subsp. *vulgatus* (O4), *B. fragilis* subsp. *vulgatus* (O15a) and *B. fragilis* subsp. *distasonis* (O15) (Table 3), suggesting that there was no correlation between the antigenic structure of *B. fragilis* and their biochemical characteristics, which confirms the earlier findings of Beerens *et al.* (1971).

In order to obtain strictly specific immune serum to each serotype, absorptions of the antisera with the cross-reacting antigens were carried out. This resulted in the removal of all the heterologous agglutinins, producing antisera specific for each individual strain (Table 4). The failure of O15a antiserum to agglutinate its homologous antigen, after being absorbed with O15 antigen (Table 3), suggests that these two strains are very closely related, if not identical. Accordingly O15a was not regarded as a distinct serotype.

Nineteen different serotypes were finally identified; ten of those belonged to subsp. *fragilis*, five to subsp. *distasonis*, two to subsp. *thetaiotaomicron* and one

Table 4. *Homologous and heterologous agglutination reactions of* Bacteroides fragilis *antigens against absorbed antisera*

		Antisera against																				
	O-antigens	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14	O15	O15a	O16	O17	O18	O19	Sal. cont.
B. f. fragilis NCTC 9343	O1	40																				
B. f. fragilis NCTC 10584	O2		40																			
B. f. theta NCTC 10582	O3			40																		
B. f. vulgatus NCTC 10583	O4				40																	
B. f. fragilis NCTC 9344	O5					40																
B. f. fragilis NCTC 8560	O6						320															
B. f. fragilis NCTC 10581	O7							80														
B. f. ovatus ATCC 8483	O8								40													
B. f. distasonis ATCC 8503	O9									160												
B. f. fragilis	O10										160											
B. f. fragilis	O11											80										
B. f. fragilis	O12												160									
B. f. distasonis	O13													40								
B. f. distasonis	O14														80							
B. f. distasonis	O15															80						
B. f. vulgatus	O15a																—					
B. f. fragilis	O16																	40				
B. f. fragilis	O17																		160			
B. f. theta	O18																			80		
B. f. distasonis	O19																				80	

each to subsp. *vulgatus* and subsp. *ovatus*. *B. fragilis* subsp. *fragilis* serotypes appear to outnumber those of the other subspecies. This may be due to the fact that 75% of the strains initially tested belonged to subsp. *fragilis*.

The association of *B. fragilis* with infection in man is well recognized, but little is known of the different serotypes which cause these infections. It is hoped that this serological study may provide a basis for a rapid and accurate identification and classification of *B. fragilis* group. Ultimately this will help us to understand the epidemiology and pathogenesis of *B. fragilis* infections.

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